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EFFECT OF BENZENE HEXACHLORIDE INSECTICIDE ON SPROUTING SEEDS

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In 1947, a new insecticide, benzene hexachloride ( $C_6H_6Cl_6$ ), was tested for use against wire worms at the experimental base of the All Union Academy of Agricultural Sciences imeni V. I. Lenin at Gorki-Leninskiye. Owing to the fact that the preparation must be introduced directly into the soil, the question arose as to whether it would have a harmful effect on sprouting plants if it came into close contact with them. Special experiments to investigate the effect of benzene hexachloride on sprouting seeds in a series of field cultures were carried out by A. A. Avakyan.

Seeds were subjected to its influence by being dusted with insecticide powder (made up of 7 - 10 percent benzene hexachloride, in talc as a filler) and mixed with it. In a majority of cultures the processed seeds showed sharply altered sprouts. The most typical feature was a delay in growth compared with the control seeds and the formation of characteristic round tumor-shaped swellings on the roots. The external appearance of the sprouts recalled the illustrations of seeds which had been processed with colchicine. This resemblance of our experimental sprouts to those treated with colchicine made us decide to subject them to microscopic analysis.

Five cultures were included in this research: two types of soft wheat (Kooparatorika and Sagna), one type of hard wheat (Kolchoznaya Tsvetaya), Belaroye millet, and Triumph beans. Except for the beans, they all showed tumors on the roots when treated with benzene hexachloride. The bean roots

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only differed from the controls by a slight retardation of growth; no tumors were formed; and in general they had a healthy, fresh appearance unlike the roots of other treated cultures.

Treatment of the cytological material was carried out using the normal microtome technique. Navashin's method was used for fixing, and Howton's, for staining gentian violet. The microtome sections were made thick (no thinner than 16  $\mu$ ) in order to avoid cutting the chromosomes into several parts; this is particularly important for the very long ribbon-shaped chromosomes of wheat. The primary roots which were in direct contact with particles of insecticide were subjected to analysis.

Microscopic analysis revealed large changes in every culture examined. When viewed through the microscope, all three wheats and the millet were very similar; the bean root differed.

The first thing apparent when looking at the preparation was the complete absence of fission, even with a sizeable or sometimes (particularly with beans) very large number of divided cells. The second, and no less striking, was the abnormal and scarcely mutilated quiescent nuclei.

Metaphase control plates of Kolkhoznyaya Tvardaya [hard] wheat (28 chromosomes) and Smena (42 chromosomes) are shown in Figures 1a and 2a. Nothing resembling the control was observed in roots treated with benzene hexachloride. Only in a few cells did the ribbon-shaped wheat chromosomes preserve their typical form (Figure 2b). As a rule, the divided cells contained chromosomes which bore a far greater resemblance to reduction rather than somatic cells; for the same diameter they were 3 - 4 times shorter than the normal somatic wheat chromosomes. Their exact calculation was generally not possible because, instead of forming a normal equatorial plate, they were located on different planes, sometimes in the center and sometimes dispersed over the whole cell. An approximate calculation gave large variations in the number of chromosomes. In the case of Kolkhoznyaya Tvardaya, some cells had less than 28, and some had far more. Cells which had been partly strangled gave the impression of being literally packed with chromosomes, which were calculated at about 100 or more. Anaphases were completely absent, and nothing resembling a spindle was observed. As with the action of colchicine, the mechanism of the achromatic system was evidently destroyed. The fission of chromosomes occurred in its turn, and their orientation in the equatorial plane and subsequent separation were also not observed. Cell fission was either absent or at least retarded.

Three separate and normally univided processes which follow one after the other -- the splitting of chromosomes, their dispersion toward the poles of the cell, and cytokinesis -- evidently had different reactions to a toxic agent, the first being by far the most stable. The presence, in divided cells, of chromosomes of a different length led one to believe that the increase in their number is not only caused by the splitting of each chromosome but also by their transverse cutting. The group of chromosomes in the center of a cell began, without breaking up, to undergo telophasic changes and was gradually brought to a state of rest, forming one large nucleus, often multilobed and having the most varied and exotic forms (Figure 2d).

In other cases, chromosomes are separated into several groups, completely irregular and containing different numbers of chromosomes (Figure 1c). Each of these groups is eventually transformed into an independent nucleus. Hence, a nucleus in quiescent cells has several different sizes and forms (Figure 2d). It can be seen from Figures 2c and 2d that the general picture of quiescent meristem cells belonging to the control differs sharply from that of the experimental shoots.

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In a state of rest, the separate abnormal nuclei of multicornel cells sometimes merge together two or three at a time. It is difficult to say whether this phenomenon, which is quite foreign to a normal wheat meristem, is caused by the incompleteness of separate nuclei or by the direct action of a nucleus on a quiescent cell.

In any case it is an undoubted characteristic of the pathological composition of tissue and the reaction of a cell to abnormal conditions. Cytokinesis either does not occur, in which case a cell remains multinuclear or polyploid, or it occurs in an abnormal manner. Since the achromatic form is disrupted, the fragmoplast is not formed, and the cell is split up in various directions by several partitions which cut it into several sections. Each of these daughter cells contains a different number of chromosomes and may be completely without a nucleus. It may be assumed that by these means cells are produced which contain a smaller number of chromosomes than is normal.

Such is the effect of benzene hexachloride on the meristem cells of hard and soft wheat. As in the action of colchicine, the most noticeable processes are the polar migration of chromosomes and the division of cells. While the splitting processes of each chromosome were still in progress the cells were either paralyzed or completely destroyed.

The reaction of millet to benzene hexachloride was basically similar to that of the three experimental wheat shoots. Quiescent nuclei showed an even greater contrast with the control because, normally, the quiescent cells of the meristem of millet roots are not only mononuclear but also mononucleolar, and such experimental shoots give a variegated picture of cells which differ in the number of nuclei and nucleoli and in size and form (Figure 3b). Figure 3 shows a normal somatic millet plate (a) and the metaphases of experimental plants (c and d). Changes which were characteristic of wheat were repeated here. However, instead of long, winding chromosomes, there were indefinitely large numbers of short, spiral, almost round, chromosomes located on different planes. The presence of bivalent chromosomes in certain cells was also new. The similarity with myotic chromosomes was in this case complete; the chromosomes were not only shortened and flattened, but also formed characteristic myotic figures (Figure 3d).

During the time that the chromosomes in experimental shoots were dividing into daughter chromosomes, the action of fungicide on millet disrupted the function of the centromere. The divided halves of the chromosomes remained joined by one undivided centromere and hung on it, forming the cross-shaped figures typical of meiosis.

Thus, compared to wheat, millet is more sensitive to the action of fungicide. There is still one uninterrupted process in it, namely, the division of the centromere.

Of all the experimental plants, beans appeared to be the most stable. The action of benzene hexachloride not only produced no tumors, no retardation of growth or injury to the seeds, but even had a certain stimulating effect which was manifested by a considerable increase in the number of mitoses in the roots compared with those of the control. The quiescent cells of the experimental plants were mononuclear, or, more rarely, dinuclear with rounded nuclei of a normal form. In metaphases, chromosomes are placed more or less in one plane, so that in a majority of cases their calculation is not difficult in spite of the fact that the increase in their number may be very considerable. Figure 4 therefore gives much more factual microscopic views than Figures 1, 2, and 3 where the observed

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dispersion of chromosomes is in fact far more complex than in the photograph because it deals with the whole depth of the cell. Of all experimental plants, the general contraction of chromosomes reaches its limit in the case of beans; chromosomes are converted from elongated bacilli to spherical shaped corpuscles (Figure 4a and b). They vary greatly according to the number of their meristem cells; the range of variation is from the normal number ( $n = 22$ ) to a high degree of polyploidy in which the number of chromosomes are by no means always multiples of the basic number.

The bean was the only plant among those investigated in which the processes of dispersion of chromosomes to the poles were not completely paralyzed. Examples of anaphases, although in a small quantity compared with the enormous number of divided cells, were found in all investigated plants. After the appearance of duonuclear cells, a further division formed plates which touched or intersected one another or joined themselves into one general plate. Another peculiarity about bean metaphases was a particular dispersion of chromosomes in the form of a ring or half ring as if some unseen spherical body were being surrounded by chromosomes (Figure 4d).

Havas (1) described how, when examining the action of colchicine on wheat and onion shoots, he noticed the presence in the cell of a certain acidophile central mass around which the chromosomes were dispersed. The possibility of the presence of a similar body in bean cells treated with benzene hexachloride cannot be excluded, but it would only have been possible to observe it visually by using corresponding colorations.

When considering all the data so far described pertaining to the effect of benzene hexachloride on the cells of our five experimental plants, it can be said that in the case of the most stable culture, beans, the processes of cytokinesis were basically affected. the migration of chromosomes to the poles of a cell was interrupted, and the function of chromosome multiplication was preserved. In the case of wheat, both the cytokinesis and polar progression of chromosomes were paralyzed, with no change in the process of their division. In the case of millet, not only were the cytokinesis and polar progression interrupted but also, in part, the processes of chromosome division; this latter was manifested by a retardation in the division of the centromere.

If it is assumed that the changes in roots described above also occur at points in their bodies, the natural consequence of these changes will be a different number of chromosomes in separate cells, tissues, organs, and whole plants.

Study of the nuclear anomalies connected with the action of colchicine on a plant cell has been one of the favorite themes for cytological research during the past 10 years. After the work of Blakeale and Avery (2), colchicine was for a long time considered as a specific material by which changes leading to polyploidy could be produced in a cell. However, when the praise of colchicine grew, the authors began research in which they attempted to connect its action with the peculiarities of its chemical composition and investigated new "polyploidogenic" substances on the principle of similar molecular structure.

In chemical composition, colchicine closely resembles the sex hormones and carcinogenic hydrocarbons which produce sharp changes in the processes of cell fission. Hence it was presumed that the specific action of colchicine on a plant depended on its structure as a polycyclic hydrocarbon (3). Other compounds made up of a series of polycyclic hydrocarbons were also examined and new, biologically active substances were found with an action

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on a cell similar to that of colchicine (4). Hence it was assumed that the specific activity of colchicine and certain other new polyploidogenic substances is the result of the presence of amino groups in their molecule, and does not depend on their polycyclic structure. New preparations with similar actions were prepared, whose chemical similarity to colchicine was this time based not on their polycyclic structure, but on the presence of amino groups (5). Aurantia was applied, and also a series of aniline dyes whose effect on a cell, similar to that of colchicine, had been studied by Dustin and his colleagues in connection with questions of animal pathology long before the discovery of colchicine (6). These research analysts were the first to study nuclear poison and colchicine, about which Havas (1) writes that it appeared to be a substance which was no more specific for producing cytokinesis than many arsenic compounds.

Alkaloids comprise an isolated group of polyploidogenic substances. Both colchicine veratrine substitutes (7) and apiole (8, 9) have been studied and produced from them.

Still another group of substances, similar in their biological action to colchicine, is formed by the mercury compounds, which are used as mordant dyes for seeds and insecticides. NIUF (10) produces both Cyanaran-ethyl mercury chloride and Ceresan-ethyl mercury phosphate (11).

Thus, the number of colchicine competitors constantly increases, and they have been supplemented by substances of a diverse origin and structure. It can no longer be said that the activity of colchicine is due to the presence of benzene rings or amino groups or other peculiarities of its molecule. A number of compounds which have neither a cyclic structure nor amino groups were produced as substances possessing the qualities required to interrupt cell fission and promote the formation of polyploid cells. Among them are substances with a very simple structure such as chloral hydrate, ether, ethyl alcohol, etc.

Research by Ostergren and Levan (12, 13) dealt the final blow to the colchicine monopoly. Levan (14) studied the effect on an onion root's meristem cells of 40 metals in the form of simple compounds, for the most part nitrates. Each substance was studied in 10 - 16 concentrations, from lethal to ineffective. An unexpected result was that all substances examined showed at least the partial mitotic disruption typical of colchicine. Complete colchicine mitosis was obtained in various concentrations of the following metals: lithium, beryllium, sodium, potassium, chromium, iron, cobalt, nickel, copper, arsenic, rubidium, yttrium, palladium, cadmium, barium, lanthanum, cerium, neodymium, erbium, gold, mercury, thallium, lead, bismuth, and thorium.

In many cases the limits of activity of these new polyploidogenic substances were considerably lower than they are in colchicine. For example, copper nitrate, yttrium sulfate, lanthanum nitrate, gold chloride, and lead nitrate become active with concentrations of 0.000,005 - 0.000,05 molecular weight. The limits of activity are generally lowered with an increase in molecular weight. In the 5th, 6th, and 7th groups of the periodic system the limits are below 0.000,05 whereas in the 2d and 3d many limits reach 0.05 - 0.2.

All salts which feed plants have high limits. The tumors which normally accompany colchicine mitosis when treatment by organic compounds is used are not always observed in similar cases using inorganic substances, but the various interruptions and retardations of growth are normal.

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Work by Löwen and Ostergren has shown that the activity of polyploidogenic substances does not depend on their chemical properties. To clarify the mechanism of their action, Ostergren produced the lipid theory, which is based upon the relationship existing between the polyploidogenic activity of a substance and its solubility in lipoids. The greater or smaller activity of a substance appeared to be caused by a purely physical property, solubility, since a positive correlation was found for solubility in lipoids and a negative correlation for solubility in water.

All the active substances investigated by the various authors produced similar changes in plant cells. Polyploidogenic activity mainly depends on the penetration of the substance into the cell and its dissolution in the necessary concentration in the lipoids. Once this dissolution has taken place, cell reaction sets in, independent of the chemical nature of the activating substance.

Everything that has been said above is in sharp contradiction to the accepted ideas on colchicine or any other specific polyploid producer. It makes one regard colchicine mitosis as the direct reaction of a cell to the injection of poison, and as a protective mechanism which can be compared, perhaps, with the universal protective process of inflammation in an animal organism.

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